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Review

Metabolism of dietary soy isoflavones to equol by human intestinal microflora – implications for health

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Soy isoflavones have received considerable attention. Individuals with isoflavones-rich diets have significantly lower occurrences of cardiovascular disease, osteoporosis, and some cancers. The clinical effectiveness of soy isoflavones may be a function of the ability to biotransform soy isoflavones to the more potent estrogenic metabolite, equol, which may enhance the actions of soy isoflavones, owing to its greater affinity for estrogen receptors, unique antiandrogenic properties, and superior antioxidant activity. However, not all individuals consuming daidzein produce equol. Only approximately one-third to one-half of the population is able to metabolize daidzein to equol. This high variability in equol production is presumably attributable to interindividual differences in the composition of the intestinal microflora, which may play an important role in the mechanisms of action of isoflavones. But, the specific bacterial species in the colon involved in the production of equol are yet to be discovered. Therefore, future researches are aimed at identifying the specific bacterial species and strains that are capable of converting daidzein to equol or increasing equol production.

Keywords: Equol / Intestinal microflora / Isoflavone / Metabolism / Pharmacological activities

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1 Introduction

Studies have shown that individuals with soy-rich diets, in which the health-promoting isoflavones are particularly abundant, have significantly lower occurrences of cardiovascular disease, osteoporosis, and some cancers such as breast, prostate, and colon cancers, in comparison with individuals with low soy diets [1]. Epidemiological studies have revealed that Asians, who consume a traditional diet high in soy products, have relatively low incidences of breast and prostate cancers, while the incidences are much higher in the Western world [2]. The incidence of breast cancer varies worldwide, with the rate of Asian women only being a third to a half of that for Caucasian women [3]. The incidence of hot flushes in Asian women is markedly lower than in Western women [4, 5]. In Western nations the prevalence of vasomotor symptoms is in the range of 60–85%, whereas only 57% of Malaysia women, 25% of Japanese women, and 18% of Chinese women suffer from hot flashes [5–7]. When Asians migrate to Hawaii or mainland North America and adopt a Western diet, they are at higher risks of breast and prostate cancers, suggesting that dietary and environmental factors, rather than racial characteristics, are involved [8]. Hedlund *et al.* [9] suggested that dietary soy provides sufficient levels of isoflavones to reduce the proliferation of normal and malignant prostatic epithelial cells and prostate cancer risk in many soy consumers. Soy isoflavones have received much attention as dietary components having an important role in reducing breast and prostate cancers [2, 4].

Isoflavones belong to a group of compounds known as flavonoids that share a basic structure consisting of two benzene rings (A and B) linked through a heterocyclic pyrone C ring. The benzenoid B ring position of the isoflavones is in the 3-position, which is different from the 2-position of flavones [2]. Isoflavones are a class of nonsteroidal estrogens that bear similarity in chemical structure and properties to estrogens. However, isoflavones show conformational binding to the estrogen receptor that classifies them as a natural selective estrogen receptor modulators (SERMs) rather than estrogens [4, 10–12] and have estrogenic or antiestrogenic effects depending on the con-

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Abbreviations: eNOS, endothelial nitric oxide synthase; ***O*-DMA,** *O*-desmethylangolensin; **SHBG,** sex hormone binding globulin

centration of endogenous estrogen and the amount and type of estrogen receptors [7, 13, 14].

At first appearance, it may seem that the answer to reducing the risks of these diseases lies in eating more soy or soy isoflavones, but in fact, it may be more complicated than this [15]. Nutritional studies have revealed greater complexity to the paradigm that dietary soy protects against these diseases, *e.g.*, prostate cancer [9]. The positive health effects of soy isoflavones have not been proven unambiguously yet. Only about 41 of 70 clinical studies showed a significant beneficial potential of soy and/or soy isoflavones [10]. *In vivo* studies have shown variations in health benefits of isoflavones among individuals, which have been attributed to dissimilarities in the populations of colonic bacteria responsible for isoflavone conversion [16, 17]. Although the soy isoflavone daidzein is reported to be less active than genistein *in vitro*, this is probably an invalid comparison. Unlike genistein, daidzein can be metabolized by the intestinal microflora and converted to dihydrodaidzein, *O*-desmethylangolensin (*O*-DMA), equol (7-hydroxy-3-[4′-hydroxyphenyl]-chroman) or 4-hydroxyequol, significantly altering its biological properties [15]. In addition to other factors, the bioavailability of soy isoflavones strongly depends on the activity of intestinal bacteria and the intestinal microflora plays a crucial role in the metabolism of isoflavones, but the underlying interactions remain poorly understood [18]. Each person consuming soy may be producing different metabolites, and this is likely to create significant variation in the potential health benefits [9]. Not all individuals consuming isoflavones produce equol [15], the most biologically active metabolite. The fact that only approximately one-third to one-half of humans possess a microflora capable of producing this metabolite has been suggested as an explanation for the conflicting results in dietary intervention studies with humans [16, 19].

Equol, named for its equine origins, was first isolated from the urine of pregnant mares in 1932 and was also present in the urine of nonpregnant mares and stallions [16]. Equol was identified in human urine for the first time in 1982 and was the first isoflavone found in human urine and blood. Its discovery led to the identification of soy as a rich source of isoflavones [20]. In 1984, Setchell *et al.* [16] first proposed that these nonsteroidal estrogens played a role in the prevention and treatment of hormone-dependent disease after high levels of the metabolite equol were found in the urine of adults consuming soy foods. Setchell *et al.* [16] postulated that the beneficial responses from soy isoflavones might correlate with the equol-producing status of individuals, suggesting a major role for equol as a biomarker for the effectiveness of soy isoflavones. Despite the potential biological importance of equol, there have been limited studies of equol effects *in vivo* because of the high cost of equol and its limited availability [21].

2 Hydrolysis of glycoside isoflavones

Almost all soy isoflavones exist as glycosides, which are less estrogenic than their respective aglycones, in soy and unfermented soy foods. Isoflavone glycosides are not absorbed intact across the enterocyte of healthy adults because of their higher hydrophilicity and molecular weights [22]. Their bioavailability requires the conversion of glycosides to aglycones *via* the action of intestinal β-glycosidase from bacteria that colonize the small intestine for uptake to the peripheral circulation [11, 16]. After ingestion, soy isoflavones are partially hydrolyzed in the small intestine [23], mostly in the jejunum [24] to release the aglycones, daidzein, genistein, and glycitein followed by absorption through the gut epithelium [19]. A considerable fraction of isoflavones, which is neither hydrolyzed nor absorbed in the small intestine, reaches the colon, together with an amount that is excreted into the small intestine through enterohepatic circulation. In the colon, the glycosylated, sulfated, and glucuronidated forms of daidzein are deconjugated by bacterial enzymes, and then absorbed or subjected to further metabolism by the intestinal microflora [6, 19, 24]. The extent of this metabolism appears to be highly variable among individuals and is influenced by other components of the diet [6].

Day *et al.* [25] investigated the ability of cell-free extracts from human small intestine and liver to deglycosylate various isoflavonoid glycosides. This study showed that human small intestine and liver had a β-glucosidase, a broad-specificity cytosolic enzyme found in abundance in the liver, kidney, and small intestine of mammals, capable of efficiently hydrolyzing various naturally occurring isoflavonoid glycosides. The intestinal β-glucosidases showed a high affinity for isoflavones, especially when the glucose residue was at position 7 of the molecule such as genistein 7-glucoside and daidzein 7-glucoside, as was the major isoflavones of most soy-containing food [20, 25]. The deglycosylation of isoflavone glycosides *via* β-glucosidase activity could be an important first step in metabolism, excretion, and biological activity, which is independent of metabolism by the colonic microflora [25].

Although the degree to which composition and function of the fecal microflora differ from mucosal microflora remains unclear, fecal samples are often used to investigate the intestinal microflora because they are easily collected [26]. Hur *et al.* [22] screened fecal bacteria from a healthy individual for the specific bacteria involved in the metabolism of soy isoflavones, and found two strains of bacteria capable of producing primary and secondary metabolites from the natural isoflavone glycosides daidzin and genistin. Both *Escherichia coli* HGH21 and the gram-positive strain HGH6 could convert daidzin and genistin to the aglycones daidzein and genistein, respectively [22].

Wiseman *et al.* [27] investigated the influence of chronic soy consumption on plasma concentrations and excretion of isoflavones and on intestinal microflora metabolism in healthy adults. The results showed that fecal β-glucosidase activity in the subjects consuming the high-soy diet was significantly higher than that in the subjects consuming the low-soy diet. The increase in β-glucosidase activity was likely to be in response to the considerable soy isoflavone glucoside consumption, suggesting that the β-glucosidase was inducible by its substrates [27].

Isoflavones have been shown to undergo enterohepatic recycling, and when administered orally, they soon appear in bile [20]. After the glucoside forms of isoflavones are partially hydrolyzed by β-glucosidases in the small intestine, the free forms appear in plasma within a short period of time between 30 min and 2 h [24]. The early rapid increase in plasma isoflavone concentration can also be explained by some initial absorption of aglycones in the stomach, duodenum, and proximal jejunum [20]. There was an early rise of genistein and daidzein concentrations in plasma within 1–2 h of isoflavone consumption, followed by a plateau and then a second peak at 4–8 h, reflecting an enterohepatic circulation [24]. Thereafter, the plasma concentration of isoflavones decreased at 12 and 24 h, after which they were not detectable at 48 h [24]. Setchell *et al.* [20] determined the pharmacokinetics of individual purified soy isoflavones in healthy subjects to assess the bioavailability of daidzein, genistein, and their respective β-glycosides. Although all isoflavones were efficiently absorbed from the intestinal tract, there were striking differences in the fate of aglycones and β-glycosides. In most subjects, the time it took to attain peak plasma concentrations after ingesting the aglycones was 4–7 h, whereas when the β-glycosides were ingested, the time was shifted to 8–11 h. Mean time for the aglycones genistein and daidzein was 5.2 and 6.6 h, respectively, whereas for the corresponding β-glycosides, mean time was delayed to 9.3 and 9.0 h, respectively, indicating that the rate-limiting step for absorption is initial hydrolysis of the glycoside moiety [20]. Setchell *et al.* [28] investigated the pharmacokinetics of the ^{13}C isotopic forms of daidzein and genistein in healthy humans, and found that the systemic bioavailability and maximum serum concentration of [^{13}C]genistein were significantly greater than those of [^{13}C]daidzein. The results also showed that serum concentrations of [^{13}C]genistein and [^{13}C]daidzein peaked after 5.5 and 7.4 h, respectively, and the bioavailability of both isoflavones was nonlinear at higher intakes, suggesting that uptake was rate limiting and saturable.

The formed free aglycones and their metabolites are absorbed and transported to liver, where they are hydroxylated and conjugated to more water-soluble metabolites such as isoflavone glucuronides and sulfates, which are eventually excreted in urine [29]. Most isoflavones in blood are glucuronide conjugate and a small amount is sulfated or unconjugated free molecules [30]. Gu *et al.* [31] studied the serum and urine isoflavone concentrations in three animal models and compared them with isoflavone profiles in women. There were significant interspecies differences in isoflavone metabolism, and the overall metabolic profile of pigs was closer to that of women than that of rats or monkeys. Monkey and rat urine contained high levels of aglycones, whereas human excreted isoflavone mainly in the form of glucuronides (>80%), with >10% as aglycones. Isoflavones in human plasma were predominantly glucuronides (75%) with 24% as sulfates and <1% as aglycones [31].

The results on the bioavailability of isoflavones in the aglycone or glucoside form in Eastern and Western human subjects are contradictory [24]. Izumi *et al.* [32] suggested isoflavone aglycones were absorbed faster and in greater amounts than their glucosides in humans. In contrast, Richelle *et al.* [23] investigated whether the bioavailability of isoflavones could be enhanced by enzymatic hydrolysis of glycosides to aglycones before consumption of a nonfermented soy food. The results showed that previous enzyme hydrolysis of glycosides to aglycones did not enhance the bioavailability of isoflavones in humans [23, 33]. Zubik and Meydani [24] investigated the bioavailability of the soy isoflavones daidzein and genistein in American women with typical American dietary habits after ingestion of the aglycone or glucoside form of isoflavones. The results showed that the bioavailability of genistein and daidzein was not significantly different when the isoflavones were consumed as either aglycone or glucoside by American women [24]. The pharmacokinetic studies by Setchell *et al.* [11, 20] showed that the bioavailability is greater when ingested as β-glycosides daidzin and genistin rather than aglycones as measured from the area under the curve of the plasma appearance and disappearance concentrations.

When equal amounts of the two isoflavones are consumed, plasma genistein concentration is consistently higher than daidzein, and this differential plasma concentration is accounted for by the more extensive distribution or further metabolism of daidzein compared with genistein [20, 24]. Vergne *et al.* [34] demonstrated that daidzein excretion was significantly lower in equol producers compared with equol nonproducers over the entire elimination period of the soy isoflavones. This difference disappeared when equol excretion was added to daidzein excretion in equol producers.

3 Metabolism of daidzein and production of equol

After the hydrolysis of daidzin and genistin, the released aglycone forms of isoflavones are either absorbed intact by the intestine or further metabolized by intestinal microflora [24]. Genistein is converted to *p*-ethyl phenol and 4-hydroxyphenyl-2-propionic acid, while daidzein is reduced to

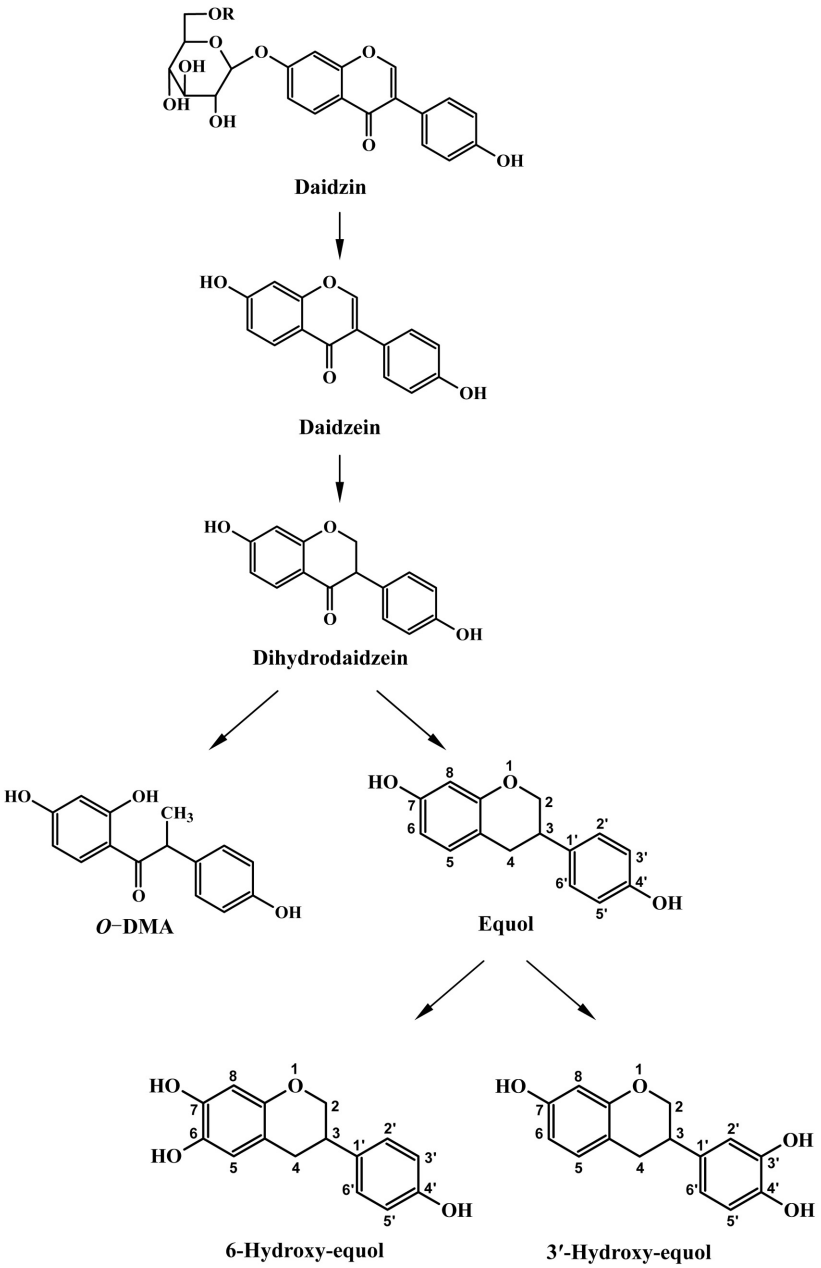

Figure 1. Metabolic pathways of daidzin.

O-DMA and equol (Fig. 1) [35, 36]. In addition, dihydrodaidzein, tetrahydrodaidzein, 3′-hydroxy-daidzein, 6-hydroxy-daidzein, 8-hydroxy-daidzein, 3-(4-hydroxyphenyl)-benzopyran-4,7-diol, and 2-dehydro-*O*-DMA have also been reported to be the metabolites of daidzein [17, 37, 38].

A large number of studies have shown that there are significant differences in biological activities between isoflavones and their bacterial metabolites. Genistein, daidzein, and equol have relatively strong affinities for estrogen receptors, while *O*-DMA has a much weaker affinity and appears to be nonestrogenic, and *p*-ethyl phenol is hormonally inert [35]. 3′-Hydroxy-daidzein has a slightly lower and 6-hydroxy-daidzein a markedly lower binding affinity than daidzein [38]. With respect to genotoxicity, it has been reported that both 3′-hydroxy-daidzein and equol are able to induce micronuclei whereas daidzein and 6-hydroxy-daidzein are not. 3′-Hydroxy-daidzein appears to act as a clastogen, possibly through redox cycling of its catechol structure, whereas equol exhibits aneuploidogenic potential [38].

Equol is absorbed more efficiently through the colon wall than daidzein [19], and appears in plasma after intake of daidzein and remains in plasma for a relatively longer period of time than do genistein and daidzein [24]. Equol concentration in plasma is negligible until 4 h and reaches a maximum concentration 24 h after the ingestion of the iso-

flavones; thereafter, equol concentration in plasma decreases but remains higher than the baseline concentrations 48 h after ingestion. There were significantly higher plasma equol concentrations in subjects after ingestion of glucoside than after ingestion of aglycone over the 48 h period [24].

Setchell *et al.* [20] compared plasma kinetics of pure daidzein and its glycosides administered as a single-bolus dose to 19 healthy women. Equol appeared in the plasma of two of the four women who consumed the glycoside daidzin. However, none of the subjects who ingested the aglycone daidzein showed equol in the plasma. It was possible that the aglycone was absorbed passively in the proximal small intestine, whereas the glycoside would not be taken up by the enterocyte, thus becoming delivered to the distal small intestine and colon for the further metabolism by bacteria [20]. Compared with the aglycone form, the glucoside form may stay in the intestines for a longer time and be subjected to both bacterial metabolism and intestinal glucosidase enzymes [24]. Zubik and Meydani [24] speculated that one possible reason for the lower concentrations of daidzein in plasma after the ingestion of glucoside than after the ingestion of aglycone is in part related to the bacterial metabolic conversion of daidzein to equol in the intestines because of a longer transit time for glucosides than for aglycones. The observed time delay of at least 6–8 h before equol appeared in substantial amounts in the plasma of equol producers would be consistent with the bacterial enzymes being of colonic origin [20].

Maubach *et al.* [39] found that equol was the predominant metabolite in breast tissue of the subjects ingesting a soy isoflavone preparation and its concentrations exceeded those in serum. The concentrations of phytoestrogens were at least 100-fold higher in urine than in serum and breast tissue. However, the subsequent studies by Maubach *et al.* [40] showed that intake of soy isoflavone supplements for five consecutive days did not result in significantly higher genistein, daidzein, and equol concentrations in breast tissue homogenate when compared with the placebo group. In urine, the concentrations of genistein, daidzein, and equol were significantly higher in the soy-supplemented subjects than in the subjects ingesting the placebo. In serum, only genistein was found to be significantly higher in the soy isoflavone group than in the placebo group. Evidently, a larger number of subjects are needed in order to establish average concentrations of soy-derived phytoestrogens in breast tissue, urine, and serum, as there was great interindividual variations in all biological matrices studied [39].

Hedlund *et al.* [41] compared plasma and prostatic fluid concentrations of isoflavones in healthy Caucasian men. The results showed that daidzein and its metabolites dihydrodaidzein, *O*-DMA, and equol were all typically present at higher concentrations in prostate fluid than plasma (median = 4–13 times that in plasma), and median levels of equol in prostate fluid were 12.7 times that found in plasma. In contrast to all other isoflavonoids, median levels of genistein were lower in prostate fluid than in plasma.

In the case in which the mother is an equol producer, it is possible that the fetus takes in equol in amniotic fluid through the recirculation system and is exposed to high concentrations of equol for a long time [42]. Todaka *et al.* [42] studied the placental transfer of phytoestrogens from mother to fetus. It is suggested that the metabolic and/or excretion rates of phytoestrogens are different between mother and fetus, and on the fetal side, metabolic and excretion rate must be low. Once phytoestrogens are transferred to the fetus, they tend to stay longer in the fetal side than in the maternal side. These results suggest that the effects of fetal exposure to phytoestrogens should be studied further [42].

Detailed studies on the metabolism of equol are scarce. Setchell *et al.* [16] suggested that equol was the end product of the biotransformation of daidzein. Once formed, equol was relatively stable and appeared to be metabolically inert, undergoing no further biotransformation. However, Rüfer *et al.* [43] recently investigated the phase I metabolism of equol. Using human liver microsomes, equol was converted to six metabolites with 3′-hydroxy-equol and 6-hydroxy-equol as main products (Fig. 1). It is suggested that phase I metabolism of equol is part of a complex biotransformation of daidzein in humans *in vivo*. This study shows that equol is a substrate for CYP enzymes and is therefore not inevitably the metabolic end product of daidzein. The formation of the aromatic hydroxylated equol metabolites may occur *via* reduction of the corresponding hydroxylated isoflavones or *via* hydroxylation of equol by CYP450 enzymes [43].

4 Diversity of equol production

4.1 Variation in equol excretion

In the typical laboratory, animal species such as mouse, rat, and monkey, due to their large cecum and abundance of microflora [16, 44], equol is produced in very large amounts and represents 70–90% of all of the circulating isoflavones [45]. For example, equol represents about 77, 52, and 80% of total serum isoflavones in rats, cynomolgus monkeys, and 6-month-old rhesus monkeys, respectively [31]. However, equol is not produced in all healthy adults in response to dietary challenge with soy or daidzein. In contrast to rodents, humans produce relatively low levels of equol [16, 45]. The extent of conversion of isoflavones to equol varies greatly among humans, presumably because of differences in the composition of intestinal microflora [46].

It has been reported that the level of urinary equol following ingestion of dietary isoflavones is highly variable between individuals. There is a 16-fold variation in total isoflavonoid excretion in urine among subjects after the high-isoflavone treatment period [47, 48]. The urinary excretion of equol varies up to 600–800-fold [46, 48, 49]. A

1527-fold variation in equol among Caucasians who consume the same amount of soy for the same length of time has been reported [15]. Most studies have demonstrated that human equol producers exhibit plasma equol concentrations ranging from ~0 to 130 nmol/L depending upon the type of diet [50]. Recent study showed that serum equol and daidzein concentrations ranged from 10.3 to 139 nmol/L (2.5–33.6 μg/L) and 16 to 1401 nmol/L (4.0–356 μg/L), respectively, whereas in urine the corresponding concentrations ranged from 16 to 12 574 nmol/L (4–3043 μg/L) and 539 to 26 834 nmol/L (137–6816 μg/L), respectively [51]. Approximately one-third to one-half of individuals are capable of metabolizing the soy isoflavone daidzein to equol and excrete substantial amounts of equol after consuming soy [7, 15, 19, 29, 36, 41, 44, 49, 50, 52–56]. However, in the study of Mathey *et al.* [57], about 59.2% of the volunteers were significant equol producers in the first experiment and 58.3% in the second. This proportion is higher than that reported in other studies but it could be due to the small size of the tested population or to the sensitivity of the ELISA technique [57].

Song *et al.* [58] assessed the prevalence of equol-producer phenotype in 91 Korean American women and girls residing in the United States and compared this with previous similarly collected prevalence data in Caucasian American women and girls. The prevalence of equol-producer phenotype was higher in Korean American (51%) than in Caucasian American women and girls (36%) [58]. Morton *et al.* [8] found that the Japanese men and women had higher concentrations of circulating daidzein, genistein, and equol than individuals from the UK. Fifty-eight percent of the Japanese men and 38% of the Japanese women had equol concentrations >20 nmol/L, compared with none of the UK men and 2.2% of the UK women [8]. Akaza *et al.* [59] focused on individuals who were able to convert daidzein into equol in Japan, Korea, and the United States, and found that the percentage of equol producers among Japanese and Korean healthy subjects was 46 and 59%, respectively, while that among the American healthy subjects was only 14%. These results indicate that compared with Western populations, Asian populations have a higher equol-producer prevalence [15, 33, 58, 59].

4.2 Equol producers and equol nonproducers

The consistent observation that not all adults are capable of synthesizing equol has led to the realization that there are two distinct subpopulations of people, defined by the terms equol producers or equol nonproducers [16, 20, 48, 60, 61]. People who have plasma equol concentrations of <40 nmol/L (10 μg/L) can be classified as "equol nonproducers;" concentrations >83 nmol/L (20 μg/L) define "equol producers." This distinction can also be derived from urine, with an equol producer defined as someone excreting >1000 nmol/L [16]. One important question concerns the demarcation between equol producers and nonproducers because equol can be found in the urine of practically all human subjects with sensitive methods [57] and no clear demarcation exists between equol producers and nonproducers. It is possible that some equol is derived from the consumption of animal food such as cow's milk [54]. Recently, Setchell and Cole [51] have developed a standardized approach, which is independent of isoflavone intake and minimizes interindividual variation in isoflavone pharmacokinetics or differences in analytical methodologies, to define equol-producer status that can be universally adopted to differentiate these two distinct populations. The study showed that the log 10-transformed urinary equol/daidzein ratio provided a clearer distinction of equol-producer status than the absolute serum or urinary equol levels. A threshold value of −1.75 provided a demarcation to define equol-producer status.

The physiologic differences between equol producers and nonproducers have not been fully elucidated [50]. Dietary modification, such as feeding wheat bran or soy protein, has been unsuccessful at changing equol-producing capability, which suggests that the intestinal microflora of an individual is relatively stable and resistant to change [27]. From many studies of repeated administration of isoflavones to the subjects, a consistent observation has shown that equol producers seem to remain "equol producers" over time, *i.e.*, "once an equol producer, always an equol producer" [16, 28]. Other researchers also suggested that unless a person was on chronic antibiotic therapy, the capacity to produce equol remained relatively stable [53, 62, 63]. In order to examine the extent to which the equol-producing ability would be stable in individual subjects, Frankenfeld *et al.* [63] evaluated concordance within an individual for the equol-producer phenotype measured at two time points (T1, T2), and found a high degree of agreement between equol-producer phenotype within an individual over a 1–3-year period. In 92 individuals, 41% were equol producers at T1 and 45% were equol producers at T2. The percentage agreement for the equol-producer phenotype was 82. Akaza *et al.* [59] measured the equol level twice at a median interval of 569 day. An 85% stability was observed in the same subjects between the two measurements. Vedrine *et al.* [64] found 1 month exposure to soy isoflavones increased significantly plasma concentration of equol for equol producers in postmenopausal women, but did not induce the ability to produce equol in equol nonproducers. These results indicate that these phenotypes are stable in most individuals over time. The stability of the equol-producer phenotype raises the possibility that the populations of equol-producing bacteria in the colon may be determined by host genetics, however, there are no data as yet to support this [53]. Although the question of whether a person who is unable to synthesize equol will ever be able to do so remains unclear [28], a ~15% of instability in equol-producing ability observed in the studies by Akaza *et al.* [59]

and Frankenfeld *et al.* [63] is thought to be meaningful, and prompts researchers to study the possibility for converting equol nonproducers to producers.

4.3 Diversity of intestinal microflora

This high interindividual variability in equol excretion is presumably attributable to differences in composition and capability of the intestinal microflora [16, 22, 29, 30, 35, 36, 47, 49, 50, 53]. The inability of some subjects to produce equol is a consequence of the lack of specific bacteria in the intestinal microflora [16, 36]. *In vitro* incubation of daidzein with fecal flora from an equol producer results in the conversion of daidzein to equol, whereas incubation with fecal flora from an equol nonproducer does not [53]. An alternative method of modifying intestinal microflora to favor equol production is through probiotic supplements. With this in mind, current efforts are aimed at identifying the specific strains of bacteria capable of converting daidzein to equol [15].

Three strains of bacteria, the gram-negative *Bacteroides ovatus* spp. and the gram-positive *Strepotococcus intermedius* spp. and *Ruminococcus productus* spp., have been identified by culturing the fecal flora from healthy Japanese adults after consumption of 70 g tofu and reported to be able to convert pure daidzein to equol *in vitro* [16, 27, 50]. Decroos *et al.* [19] isolated a stable mixed microbial culture producing equol *in vitro*, originating from a human fecal sample. The culture can be restricted to four dominant bacterial strains, *Enterococcus faecium* EPI1, *Lactobacillus mucosae* EPI2, *Finegoldia magna* EPI3, and *Veillonella* sp strain EP, but the single strain capable of transforming daidzein into equol has not yet identified. Recently, Decroos *et al.* [65] administered a mixed microbial culture (EPC4) isolated previously to the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The results show that administering EPC4 can constitute a novel means for converting an equol nonproducer into an equol producer. However, this mixture is not always present in human intestine. This explains why human subjects can be either equol producers or nonproducers. The addition of this mixed culture to a fecal culture from an equol nonproducer may stimulate equol production, indicating that equol-producing bacteria have a potential use as a probiotic for the *in vivo* stimulation of equol production [19, 65]. Minamida *et al.* [66] isolated an anaerobic gram-positive rod-shaped strain capable of producing equol from daidzein. Its 16S rDNA gene sequence (1428 bp) showed 99% similarity with that of the human intestinal bacterium SNU-Julong 732 (AY310748) and 93% similarity with that of *Eggerthella lenta* ATCC 25559T (AF292375). This strain converted daidzein to equol *via* dihydrodaidzein in an equol-assay medium anaerobically. The addition of butyric acid and arginine increased the conversion ratio of daidzein to equol 4.7- and 4.5-fold, respectively. Atkinson *et al.* [55] found that daidzein metabolizing bacteria remained viable after storage at $-180^{\circ}C$.

Although the development of antibiotics has been one of the great triumphs of modern medicine, indiscriminate use predisposes humans to opportunistic infections and will certainly exacerbate the present crisis of antibiotic resistance [67]. Treatment with antibiotics might alter plasma isoflavonoid patterns and result in a marked reduction in plasma equol concentrations [50]. However, the use of antibiotics to alter bacterial populations in the intestine could provide a potential means to identify the bacterial species responsible for converting daidzein to equol. Blair *et al.* [50] studied the effect of oral treatment with antibiotics on plasma equol concentration in cynomolgus monkeys. The results showed that equol concentrations were reduced compared with baseline by 80, 93, 98, and 99% after treatment with metronidazole, kanamycin, vancomycin, and kanamycin + vancomycin, respectively, and daidzein concentrations were increased compared with baseline by treatment with doxycycline, kanamycin, and kanamycin + vancomycin. Similar increases in dihydrodaidzein were observed after treatment with kanamycin and metronidazole. These results also demonstrated that individual antibiotics altered plasma isoflavone levels in unique patterns. Treatment with kanamycin reduced plasma equol levels while increasing plasma levels of daidzein, dihydrodaidzein, and glycitein. In contrast, treatment with doxycycline did not affect plasma equol levels, but plasma levels of daidzein, genistein, dihydrogenistein, and glycitein were elevated. It is possible that some of the antibiotics may have direct effects on isoflavone metabolism, whereas others may alter absorption through the intestinal wall [50]. The inhibition of equol production by metronidazole and kanamycin is in agreement with the data published by Atkinson *et al.* [55], who found that some antibiotics inhibited the production of equol but had no effect on dihydrodaidzein production and the conversion of daidzein to dihydrodaidzein, and of dihydrodaidzein to equol, might be carried out by different bacteria, and that the bacteria involved might differ among individuals. Rafii *et al.* [17] also suggested that different bacteria were involved in the different steps of the postulated conversion of daidzein to equol.

Wang *et al.* [68] isolated a rod-shaped, gram-negative anaerobic bacterium from human feces, named Julong 732, which was capable of metabolizing a racemic mixture of dihydrodaidzein to enantiomeric pure *S*-equol under anaerobic conditions and was not able to produce equol from daidzein, tetrahydrodaidzein, and dehydroequol. Hur *et al.* [22, 37] previously isolated a gram-positive strain HGH6 that converted daidzin to dihydrodaidzein but did not convert it further to equol, and identified another gram-positive anaerobic bacterium *Clostridium sp.* HGH136 from human feces that cleaved the C-ring of daidzein to *O*-DMA. Schoefer *et al.* [69] reported that daidzein was in part degraded to *O*-DMA by *Eubacterium ramulus*, which represented an

average of 0.16% of the total fecal flora and might therefore be one of the predominant isoflavonoid degrading bacteria in the human gastrointestinal tract. Tamura *et al.* [70] isolated a dihydrodaidzein-producing intestinal bacterium TM-40 from a 7-year-old healthy boy's feces. The strain TM-40 (AB249652), which exhibits a 93% similarity to that of *Coprobacillus catenaformis* (AB030218) and seems to be a new species, produced dihydrodaidzein both from daidzein and daidzin, but did not produce equol.

However, the specific bacterial species and environmental conditions in the colon involved in the production of equol are yet to be discovered. Although equol production has been established *in vitro* from human fecal samples, efforts to isolate bacteria that produce equol have not been successful so far [19]. The identification of the bacterial species responsible for converting daidzein to equol is of considerable importance and is a major challenge because of the large number of bacteria that reside in the colon and small intestine [16, 27].

The human intestine is more densely populated with microorganisms than any other organ and is a site where the microflora may have a pronounced impact on human health [71]. The human endogenous intestinal microflora is an essential "organ" [26] with several important intestinal functions, including nutrient absorption, mucosal barrier fortification, protection against epithelial cell injury, regulation of host fat storage, stimulation of intestinal angiogenesis, xenobiotic metabolism, postnatal intestinal maturation, regulating epithelial development, and instructing innate immunity [26, 67, 71]. The gastrointestinal tract in human is colonized by a vast, complex, and dynamic consortium of bacterial symbionts and commensals that may outnumber our somatic and germ cells, and a microbial density approaches 10^{12} organisms *per* gram in the human colon. The species composition of symbionts and commensals varies along the length of the intestine, changes as human develops and ages, and is influenced by the environment [67]. Eckburg *et al.* [26] examined 13 355 prokaryotic ribosomal RNA gene sequences from multiple colonic mucosal sites and feces of healthy subjects to understand the intestinal microbial diversity. The results showed that a majority of the bacterial sequences corresponded to uncultivated species and novel microorganisms, and discovered significant intersubject variability and differences between stool and mucosa community composition. Ley *et al.* [72] suggested that obesity might affect the diversity of the intestinal microflora and intentional manipulation of community structure might be useful for regulating energy balance in obese individuals.

4.4 Influence of habitual diet on equol production

The diet can determine the dominant bacterial strains present in the gastrointestinal tract and certain dietary changes may alter the bacterial profile of the intestine. Therefore, the habitual diet may influence the metabolism of isoflavones and the production of equol [6, 15, 33]. It has been suggested that the intestinal metabolism and bioavailability of isoflavones in humans and the rate of formation of equol are influenced by dietary habits, the food matrix, the composition of intestinal microflora, the extent of intestinal bacterial fermentation, intestinal transit time, and alterations in the redox level in the large intestine [24, 47, 48, 53, 73]. Setchell and Cole [51] found that the frequency of equol producers in vegetarians was 59%, similar to the reported frequency in Japanese adults consuming soy, and much higher than for nonvegetarian adults (25%). Higher dietary fiber and plant protein, less fat and more carbohydrate intakes have also been associated more strongly with equol producers than equol nonproducers among female subjects [52]. However, Zhao *et al.* [74] could not show any correlation between the equol excretion and intakes of these items among the Japanese female population. One of the reasons for this may be that the Japanese subjects eat soy products more or less, and therefore, the differences are reduced.

A diet rich in carbohydrates may stimulate equol production in an individual harboring an intestinal microflora which contains equol-producing bacterial species [6, 19]. Hydrogen gas and SCFAs are the major metabolic products of the fermentation of carbohydrates by the intestinal microflora. Decroos *et al.* [19] suggested that equol production was stimulated to a large extent by hydrogen gas, probably acting as electron donor in the biotransformation reaction from daidzein to equol, indicating that hydrogen gas has an important role in the mechanism of equol production. Increased equol production is also found in the presence of propionate and butyrate, suggesting that a diet rich in carbohydrates stimulates equol production [19]. Therefore, good equol producers often consume more carbohydrate as percentage of energy than equol nonproducers [48].

Tamura *et al.* [75] evaluated the prebiotic effects of difructose anhydride III, a newly manufactured nondigestible disaccharide with unique fermentation properties, on equol production and on plasma cholesterol concentrations related to the changes in equol production. The results show that difructose anhydride III can efficiently enhance plasma equol concentrations, which may be associated with an increase in equol production and a decrease in equol degradation by enterobacteria, and therefore may contribute to the hypocholesterolemic effect of difructose anhydride III.

Zafar *et al.* [76] investigated the effect of inulin, which is composed of fructooligosaccharide with a different degree of polymerization (DP), on isoflavone absorption, and did not see any significant difference in serum isoflavone concentration due to the presence of inulin. Serum equol concentrations were significantly lower in the group cofed with inulin compared to the group fed isoflavone without inulin. Decroos *et al.* [19] also found that adding fructooligosac-

charides was inhibitory for equol production. Although metabolism of fructooligosaccharides by intestinal bacteria results in a large release of hydrogen, the presence of fructooligosaccharides might alter the colonic bacterial flora and suppress the bacteria responsible for the formation of equol, and cause simultaneously a shift in hydrogen utilization. As a result daidzein is no longer transformed to dihydrodaidzein or equol [19, 76].

Hedlund *et al.* [41] suggested that the ability of Caucasian men to produce equol was favorably influenced by the long-term consumption of high amounts of soy in combination with modest amounts of meat. Stratified analyses revealed that men who had consumed ≥30 mg/day soy isoflavones for at least 2 years had 5.3 times the probability of producing equol than men who had consumed ≤5 mg/day. Those men who consumed animal meat regularly had 4.7 times the probability of producing equol than men who did not consume meat [41]. However, a significant negative correlation was found between the proportion of energy from fat in the habitual diet and urinary equol excretion, indicating that the dietary fat intake decreased the capacity of intestinal microflora to synthesize equol [48].

Lactobacillus and *Bifidobacterium* have been used as probiotics with the aim of managing intestinal disorders by improving the intestinal microbial balance. However, the administration of probiotics is likely to influence the effect of isoflavones on the host through changes in the gastrointestinal environment [77]. Bonorden *et al.* [33] and McMullen *et al.* [78] found that the intervention with consumption of probiotics *Lactobacillus acidophilus* and *Bifidobacterium longum* for 2 months did not significantly alter equol production status. It is possible that probiotic consumption alters the intestinal environment but not enough to significantly increase equol production [33]. However, Tamura *et al.* [77] suggested that *Lactobacillus gasseri* could suppress the production of equol, and significantly decrease both the plasma equol concentration and the total amount of equol present as aglycone in the cecal contents. Bacteriocins and several metabolic compounds such as organic acids, fatty acids, and H_2O_2 produced by lactic acid bacteria have antimicrobial effects. Increased numbers of lactobacilli would therefore affect the composition and/or metabolic activity of intestinal microflora [77].

5 Pharmacological activities of equol

The clinical effectiveness of soy isoflavones in cardiovascular, bone, and menopausal health may be a function of the ability to biotransform soy isoflavones to the more potent estrogenic metabolite, equol [16], which may enhance the actions of soy isoflavones, owing to its low affinity for serum proteins, greater affinity for estrogen receptors compared with its precursors, daidzein or dihydrodaidzein, and superior antioxidant activity [60].

Equol is a nonsteroidal estrogen of the isoflavan class. Unlike daidzein and genistein, equol is unique in having a chiral center due to the lack of a double bond in the heterocyclic ring, and is a chiral molecule that can exist in two enantiomeric forms, *S*- and *R*-equol. Human intestinal bacteria can exclusively synthesize *S*-equol, the naturally occurring enantiomer, from daidzein [46, 60, 61]. Equol has the strongest binding affinities and estrogenic activities especially for ERβ among the daidzin metabolites [13]. The two equol enantiomers *R*- and *S*-equol show very different behavior in terms of their binding affinities with ERα and ERβ [46]. The relative binding affinities of the *R*- and *S*-equol enantiomers for ERα were 0.47 and 2.0% with that of 17β-estradiol. *S*-equol binds ERβ ≈ 20% with as much affinity as does 17β-estradiol, whereas the *R*-enantiomer bound at ≈1% of the affinity [61]. The binding affinity of the natural enantiomer, *S*-equol, is similar in these respects to genistein, the most estrogenic soy isoflavone [46].

It has been reported that equol shows effective free fractions in serum of 49.7% [79], which is considerably greater than the proportion of free daidzein (18.7%) or estradiol (4.6%). This may effectively contribute to enhancing the overall potency of equol [16]. Maximal responses to isoflavone intake are observed in equol producers, who are at lower risk of breast cancer than equol nonproducers. The equol producers in postmenopausal women have smaller bone loss changes than equol nonproducers [80]. Retrospective analysis reveals that significant improvement of plasma lipids with the soy diet, including reductions in total cholesterol, LDL cholesterol, LDL/HDL ratio, plasma triglycerides and lipoprotein(a), may have been limited to equol producers [81].

Niculescu *et al.* [82] suggested that the capacity to produce equol might be an important modulator in responsiveness to isoflavone treatment. Isoflavones induced changes in gene expression in postmenopausal women, and the changes were related to increased cell differentiation, increased cAMP signaling and G-protein-coupled protein metabolism, and increased steroid hormone receptor activity. The response to isoflavones was different between equol producers and nonproducers, with enhanced expression of some of the estrogen-responsive genes mainly occurring within the equol producers.

5.1 Antioxidant activities of equol

Since it is believed that many of the beneficial properties of isoflavones, *e.g.*, prevention of coronary heart disease as well as breast, prostate, and colon cancers, may be related to their antioxidant activities, there has been a surge of interest in exploring the antioxidant activities of the naturally occurring isoflavones and their corresponding metabolites [83–85]. Isoflavones may have antioxidant properties through hydrogen/electron donation *via* hydroxyl groups, and therefore act as free radical scavengers [85]. The num-

ber and position of hydroxyl groups were determining factors for isoflavone antioxidant activity, with hydroxyl substitution being of utmost importance at the C-4′ position, of moderate importance at the C-5 position, and of little significance at the C-7 position [83].

The studies on the antioxidant properties of isoflavones and their metabolites indicate that the metabolism to the bacterial metabolite equol as well as the oxidative metabolites 3′-hydroxy-genistein, and 3′-, 6-, and 8-hydroxy-daidzein enhance their antioxidant properties [84]. Mitchell *et al.* [85] assessed the hydrogen-donating ability of a range of isoflavones using electron spin resonance (ESR) spectroscopy, the ferric reducing ability of plasma (FRAP) assay, and the Trolox equivalent antioxidant capacity (TEAC) assay, and showed that equol was more effective antioxidants than genistein and daidzein [85, 86]. Reducing the isoflavone nucleus to yield the isoflavan structure appears to enhance the antioxidant activities [83].

Specific structural criteria defining the free radical scavenging activities of flavonoids, including the 2,3-double bond with the 4-oxo group and the 3-hydroxyl group in the C-ring, the 5,7-dihydroxyl structure in the A-ring, and the ortho-dihydroxyl structure in the B-ring, have already been characterized [84]. Although equol lacks the 2,3-double bond, the 4-oxo group, and a 5,7-dihydroxyl structure on the isoflavone nucleus, equol and its 4-hydroxy and 5-hydroxy derivatives are the most potent antioxidants in the naturally occurring glycosidic and methoxylated forms of isoflavones, the free aglycones, and their biological metabolites [83 – 85]. The higher antioxidant activity of equol may be a result of its nonplanar structure that confers equol with a greater flexibility for conformational changes, which can enable it to penetrate more easily into the interior of the membrane and protein or lipid structures to prevent oxidative damage *in situ* than some of the other isoflavones that are more rigid in structure [83, 84].

Hwang *et al.* [86] investigated the antioxidant properties of equol on the basis of its ability to affect NO production or utilization, and found that equol possessed a strong antioxidant potential by virtue of its ability to enhance bioavailable NO through the downregulation of $O_2^{-\bullet}$ production, thereby preventing LDL modification to an atherogenic particle. Decreased $O_2^{-\bullet}$ production resulted in increased free NO levels (but not total NO production) indicating that decreased reactions between $O_2^{-\bullet}$ and NO are an outcome of equol's antioxidant activity in cell culture. The antioxidant effects of equol during J774 cells-mediated LDL modification are based on a downregulation of $O_2^{-\bullet}$ production that is achieved, at least in part, through inhibited the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [86].

Joy *et al.* [87] provided insight into the signaling pathways regulating endothelial nitric oxide synthase (eNOS) activity in human endothelium and identified equol as a potent activator of acute NO production. Equol rapidly stimulates phosphorylation of ERK1/2 and phosphatidylinositol 3-kinase/Akt, leading to the activation of eNOS and increased NO production at resting cytosolic Ca^{2+} levels. Identification of the nongenomic mechanisms by which equol mediates vascular relaxation provides a basis for evaluating potential benefits of equol in the treatment of postmenopausal women and patients at risk of cardiovascular disease [87]. NO is known to have both atherogenic and vascular protective effects, depending on the source and amount of production. NO produced by endothelial NO synthase (eNOS) has a vasodilator function and has a protective effect. However, inducible nitric oxide synthase (iNOS) in macrophages produces a large amount of NO in response to various stimuli, and potent oxidative properties of NO produced by iNOS appear to induce atherosclerosis [88]. Kang *et al.* [88] demonstrated that equol inhibited LPS-induced NO production and iNOS gene expression in macrophages and these effects were mediated, at least in part, by inhibiting Akt activation and subsequent downregulation of nuclear factor-κB (NF-κB) activity. The inhibitory effect of equol on NO production and iNOS gene expression provides a possible mechanism responsible for the antiatherosclerotic effect of equol and soy isoflavones.

Jackman *et al.* [89] compared the antioxidant effects of equol and daidzein in carotid and basilar artery of normal and hypertensive rats, and found that equol displayed antioxidant activity in the basilar artery and preserved vasorelaxant activity in carotid arteries from hypertensive rats. These results are consistent with the concept that equol may represent a useful therapeutic agent for vascular disease of both genders especially in the cerebral circulation.

Equol is found to have strong antioxidant action against acute UV A (320 – 400 nm)-induced lipid peroxidation of mouse skin. The antiphotoaging, anti-inflammatory, immunoprotective, and anticarcinogenic activities against solar-simulated UV irradiation suggest that equol can be developed as a helpful topical photoprotective agent [90, 91], *e.g.*, a potential supplementary ingredient in topical sunprotective cosmetic products for humans particularly susceptible to nonmelanoma skin cancer [92].

Magee *et al.* [93] compared the biological effects of purified *S*-equol to that of racemic equol on breast and prostate cancer cells of varying receptor status *in vitro*. The result showed that racemic equol prevented DNA damage in MCF-10A breast cells and had strong antigenotoxic activity in contrast to the purified *S*-equol enantiomer, implicating the *R*-, rather than the *S*-enantiomer as being responsible for the antioxidant effects of equol.

Choi [94] found that equol severely decreased the ratio of reduced glutathione and oxidized glutathione in primary cortical neuron cells exposed to equol, depending on the dose and time of treatment. The results indicate that chronic administration of daidzein in rats may cause an inhibition of lipid peroxidation and a decrease in glutathione concentration, suggesting that daidzein may act not only as an anti-

oxidant, but also a prooxidant in the brains of rats. The excessive use of daidzein is not likely to produce beneficial health effects [94].

Some estrogens, antiestrogens, and their metabolites have been shown to behave as antioxidants which may contribute to their beneficial effects. However, both estrogens and antiestrogens can be metabolized to phenoxyl radicals, quinones, and semiquinone radicals, all of which can cause damage in cells either through alkylation or oxidation of cellular macromolecules including DNA [95]. It has been reported that 3′-hydroxyequol with catechol structure is the main metabolite of equol [43]. The catechols may undergo redox cycling after oxidation to semiquinones to form the corresponding quinones and reactive oxygen species, both of which can damage cellular macromolecules and cause cytotoxicity and genotoxicity. All these aspects of the oxidative metabolism of isoflavones deserve further investigation [96]. The observation that major human metabolites of daidzein exhibit estrogenic and genotoxic potential may be of relevance for the safety evaluation of isoflavones [38].

5.2 The regulation effects of endogenous hormones

Soy isoflavones have structures similar to that of estrogen and have received attention as alternatives to hormone replacement therapy for the prevention of postmenopausal osteoporosis [80]. The binding affinity of equol for ERα and ERβ is similar to that of genistein and equol induces transcription more strongly than any other isoflavone, especially with ERα [97].

The previous studies have suggested that consumption of isoflavone-rich foods and increased urinary excretion of equol have been associated with a reduced risk of breast cancer [3, 49, 98]. The inverse association between urinary equol excretion and breast cancer risk may not have been wholly attributable to differences in isoflavone intake, but rather to differences associated with the ability to produce equol [49, 98]. The association of equol excretion and lowered breast cancer risk may largely reflect the tendency of equol producers to have more favorable hormonal profiles, as opposed to merely reflecting increased isoflavone intake [49].

Equol itself may exert beneficial effects on the regulation of endogenous hormones [49]. The ability to produce equol might represent colonic bacterial enzyme activity that increases fecal steroid excretion [49, 53]. Urinary equol excretion has been inversely correlated with circulating free estradiol, and positively correlated with sex hormone binding globulin (SHBG) [53, 99]. Bonorden *et al.* [33] hypothesized that the ability to convert daidzein to equol was characteristic of bacteria that also reduced enterohepatic circulation of reproductive hormones and might explain the inverse association between equol excretion and breast cancer risk. Equol producers generally have a hormonal pattern overall consistent with lowered breast cancer risk, such as lower concentrations of estrone, estrone-sulfate, prolactin, testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEA-sulfate, and cortisol, and higher concentrations of SHBG and midluteal progesterone, as well as trends toward longer menstrual cycle and phase lengths, when compared with equol nonproducers [49]. However, Bonorden *et al.* [33] found a nonsignificant tendency toward lower estrone, estrone-sulfate, and testosterone, and higher SHBG concentrations in premenopausal equol producers. Frankenfeld *et al.* [100] evaluated concentrations of serum hormones, SHBG, and urinary estrogen metabolites in relation to daidzein-metabolizing phenotypes in postmenopausal women. No appreciable differences in serum hormone concentrations in relation to equol-producer phenotype were observed. The result suggested that interindividual variability in intestinal bacteria might be related to differences in products of hormone metabolism in postmenopausal women. The result differed from those of Duncan *et al.* [49] because postmenopausal women, compared to premenopausal women, had lower circulating concentrations of several sex hormones and SHBG, which, along with the women in the study being overweight, might have reduced the variation in serum hormones [100].

Although estrogens can stimulate the proliferation of cancer cells, their metabolites may be important in the development of breast cancer. Certain metabolites of estradiol or estrone can directly produce DNA damage in target tissues, independent of their interaction with the estrogen receptor [101]. These metabolites are generated by two major pathways: formation of catechol estrogens (2-hydroxyestradiol, 2-hydroxyestrone, 4-hydroxyestradiol, and 4-hydroxyestrone) and, to a lesser extent, 16α-hydroxylation (16α-hydroxyestrone) [101, 102]. If catechol estrogens are oxidized to the electrophilic catechol estrogens-quinones, they may react with DNA. Specifically, the carcinogenic 4-hydroxyestradiol and 4-hydroxyestrone are oxidized to estradiol-3,4-quinone and estrone-3,4-quinone, which can react with DNA to form predominantly depurinating adducts. These adducts are released from DNA to generate apurinic sites. Increased levels of these quinones and their reaction with DNA occur when estrogen metabolism is unbalanced. Error-prone base excision repair of this damage may lead to the mutations that can initiate breast, prostate, and other types of cancers [102].

16α-Hydroxyestrone can covalently bind to the estrogen receptor and also increase unscheduled DNA synthesis. The mechanism by which 16α-hydroxyestrone directly or indirectly damages DNA remains unknown. The 16α-hydroxylation of estrone has been found to be approximately 50% greater in postmenopausal patients with breast cancer than in healthy control subjects. An increase is also detected in healthy women at high risk to develop breast cancer [101]. Some epidemiologic studies report an association between

a low ratio of urinary 2-hydroxyestrogens (2-hydroxyestradiol + 2-hydroxyestrone) to 16α-hydroxyestrone and increased breast cancer risk [103]. Atkinson *et al.* [53] found that equol excretion, but not total isoflavone excretion, correlated positively with the 2-hydroxyestrone: 16α-hydroxyestrone ratio and suggested that the colonic bacterial profile associated with equol production might be involved in estrogen metabolism, and therefore possibly influence breast cancer risk. Nettleton *et al.* [103] also found lower urine 2-hydroxyestrone/16α-hydroxyestrone ratios in women with breast cancer and suggested that soy consumption increased this ratio only in women who were equol producers.

In addition, Fujioka *et al.* [80] demonstrated that equol had the ability to inhibit bone loss *in vivo* without estrogenic effects on the reproductive organs in ovariectomized mice. Uesugi *et al.* [104] also found that equol offered benefits to reduce effectively on bone resorption enhanced by menopause and improve significantly climacteric hot flash and hypertension. Wu *et al.* [105] determined the effects of isoflavone intake and walking and their interaction on bone and lipid metabolism in postmenopausal women. The combination of isoflavones and exercise exhibited favorable effects on serum lipid and body composition of postmenopausal women. It is suggested that the preventive effects of isoflavones on bone loss depend on the intestinal microflora for equol production [105]. Therefore, it is important to promote or activate intestinal microflora that produce equol to obtain the maximal effects of isoflavones on prevention of bone loss when estrogen status is deficient [80]. Kang *et al.* [106] examined the effect of equol on tumor necrosis factor-α (TNF-α) gene expression to elucidate a possible mechanism by which equol exerted osteoprotective effect. The results demonstrate that equol inhibits LPS-induced TNF-α gene expression in macrophages and these effects are mediated, at least in part, by inhibiting NF-κB activity, and suggest a direct application of equol as alternative or supplement of hormone replacement therapy, especially for equol nonproducers.

Hwang *et al.* [13] reported that equol inhibited the osteoclast formation in the significant manner. Acute clinical responses to soy are observed within the good equol producers, who exhibit the least severe menopausal symptoms. The bone mineral density of lumbar spine increased significantly by 2.4% in postmenopausal women capable of producing equol, while there was no significant change in bone mineral density levels for equol nonproducers [13].

Soy isoflavonoids have well-established estrogenic properties, raising concerns that high isoflavonoid intake may promote development of uterine and breast cancers. To address this concern, Wood *et al.* [107] evaluated the effects of high-dose racemic equol (1020 mg/day) on reproductive tissues in female cynomolgus monkeys for approximately 1 month, and found that uterine weight, endometrial thickness, glandular area, and epithelial proliferation in the uterus were not significantly different among treatment groups, indicating that high doses of isoflavonoids had minimal uterotrophic or mammotrophic effects in an established postmenopausal primate model.

5.3 Antiandrogenic activities and the prevention of prostate cancer

The previous studies correlate the high consumption of isoflavones with the low incidence rates of benign prostate hyperplasia and prostate cancer in Asian men compared with Western men [108]. It has been found that daidzein and its metabolites (but not genistein) are typically present at higher levels in prostate fluid than plasma [41], strongly suggesting an ability of the prostate to concentrate these weak estrogens [109]. On average, daidzein is concentrated 2.2-fold, and equol is concentrated 45-fold in prostatic fluid [15]. The concentration of equol is higher in both the plasma and prostatic fluid of men from Hong Kong than from men in the UK and Portugal. Equol is 17 times higher in prostatic fluid samples from Hong Kong than those from Britain, and five times higher than in samples from Portugal [109]. The high concentrations of equol in prostatic fluid increase the potential for equol to have direct effects in the prostate [41]. Therefore, it is possible that lifelong exposure to isoflavones may play a significant role in the low incidence of prostate cancer in Chinese and other Asian men [109]. Hedlund *et al.* [15] provided strong evidence that equol had potent inhibitory effects on the proliferation of benign and malignant prostatic epithelial cells, and suggested that the intestinal conversion of daidzein to equol was responsible, at least in part, for the reduced risk of developing prostate cancer. The clinical research has identified that equol nonproducers are at higher risk for prostate cancer than are equol producers [59, 108].

Akaza *et al.* [59] conducted a case-control study for the comparisons of percent equol producers between prostate cancer patients and controls in Japanese, Korean, and American residents. The results show that the percentage of equol producers among patients and controls is 29 and 46% in Japan, 30 and 59% in Korea, and 17 and 14% in the United States, respectively. The results suggest that the ability of producing equol or equol itself is closely related to the lower incidence of prostate cancer and the percentage of equol producers is significantly lower among patients with prostate cancer [59].

Lund *et al.* [108] examined the effects of equol on prostate growth and luteinizing hormone secretion, and found that equol reduced ventral prostate and epididymal weight and increased circulating luteinizing hormone levels. The beneficial effects of soy in relation to prostate health may be due to the unique antiandrogenic properties of equol, rather than its estrogenic properties. The antiandrogenic properties of equol are unique in that equol specifically binds 5α-dihydrotestosterone, but not testosterone, dehy-

droepiandrosterone, or the androgen receptor with high affinity, and thereby prevents 5α-dihydrotestosterone from binding the androgen receptor. The blockade of androgen action can be beneficial for preventing growth of reproductive tissues with 5α-dihydrotestosterone dependency such as prostate and epididymis [108]. Lephart [110] suggested that equol could significantly reduce ventral prostate weight by presumably binding to 5α-dihydrotestosterone and might be an effective treatment in addressing women's and men's health issues associated with aging as well as other steroid hormone-dependent disorders, such as male/female pattern baldness, facial and body hair growth, skin health, skin integrity, and emotional and mental health, *etc*. [108, 110].

Rannikko *et al.* [111] evaluated the effects of isoflavones on hypothalamic-pituitary-testicular axis in prostate cancer patients and concluded that short-term treatment with isoflavones interfered with the hypothalamic-pituitary-testicular axis by inducing partially compensated primary hypogonadism and testicular resistance to luteinizing hormone. During the course of treatment, serum concentration of equol correlated strongly with the concomitant decrease in serum androgen bioactivity. Although the potential antiandrogenic effects of equol have been suggested, it is unlikely that equol is the only contributor to these results. A combined effect is suggested by Rannikko *et al.* [111]. Genistein (and possible other isoflavones) may inhibit LH-stimulated testosterone secretion, and equol may sequester 5α-dihydrotestosterone from the androgen receptor and therefore block the intracellular effects of 5α-dihydrotestosterone. The results support a possible role for isoflavones in the prevention of prostate cancer [111].

6 Conclusions

Soy isoflavones are biologically active in humans and have received considerable attention. Individuals with isoflavones-rich diets have significantly lower occurrences of cardiovascular disease, osteoporosis, and some cancers such as breast, prostate, and colon cancers. Maximal responses to isoflavone intake are observed in people who are good equol producers. The clinical effectiveness of soy isoflavones may be a function of the ability to biotransform soy isoflavones to the more potent estrogenic metabolite equol, which may enhance the actions of soy isoflavones, owing to its greater affinity for estrogen receptors, unique antiandrogenic properties, and superior antioxidant activity.

The higher antioxidant activity of equol may be a result of its nonplanar structure that confers equol with a greater flexibility for conformational changes, which can enable it to penetrate more easily into the interior of the membrane and protein or lipid structures to prevent oxidative damage *in situ* than some of the other isoflavonoids that are more rigid in structure. The association of equol excretion and lowered breast cancer risk may largely reflect the tendency of equol producers to have more favorable hormonal profiles, as opposed to merely reflecting increased isoflavone intake. The antiandrogenic properties of equol are unique in that equol specifically binds 5α-dihydrotestosterone with high affinity, and thereby prevents 5α-dihydrotestosterone from binding the androgen receptor, and is beneficial for preventing growth of reproductive tissues with 5α-dihydrotestosterone dependency such as prostate and epididymis.

However, not all individuals consuming daidzein produce equol. Only approximately one-third to one-half of the population is able to metabolize daidzein to equol. This high variability in equol production is presumably attributable to interindividual differences in composition of the intestinal microflora, which may play an important role in the mechanisms of action of isoflavones because the intestinal metabolism of isoflavones largely determines the levels of circulating isoflavones and their metabolites. The inability of some subjects to produce equol is a consequence of the lack of specific components of the intestinal microflora.

An alternative method of modifying intestinal microflora to favor equol production is through probiotic supplements. The identification of the bacterial species responsible for converting daidzein to equol is of considerable importance and is a major challenge because of the large number of bacteria that reside in the colon and small intestine. However, the specific bacterial species and environmental conditions in the colon involved in the production of equol are yet to be discovered. Although equol production has been established *in vitro* from human fecal samples, efforts to isolate bacteria that produce equol have not been successful so far. Therefore, future researches are aimed at identifying the specific bacterial species and strains that are capable of converting daidzein to equol or increasing equol production. It is possible that the consumption of equol-producing bacteria as a probiotic can alter the intestinal environment and significantly stimulate equol production.

In addition, equol for dietary administration may be prepared from daidzein, which is readily available in large quantities from soy, especially soy hypocotyls, by the separated equol-producing bacteria or based on transfer hydrogenation as well as a biomimetic synthesis [46]. Heemstra *et al.* [112] recently described the first enantioselective total synthesis of *S*-equol, utilizing a route that was brief, cost effective, and scalable. Future work will focus on optimizing yields and selectivity through screening of both reagents and reaction conditions.

7 References

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